JC18 Rec'd PCT/PTO 05 NOV 2001

FORM PTO-1390 (Modified) (REV 11-2000)

U S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: **RU-0170**

U S APPLICATION NO (IF KNOWN, SEE 37 CFR: **10/009472**

INTERNATIONAL APPLICATION NO	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/US00/11893	**2 May 2000**	**4 May 1999**

TITLE OF INVENTION
Compositions and Methods for Detection of Active Proteases

APPLICANT(S) FOR DO/EO/US
LAM, Eric et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S C 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C 371
3. ☐ This is an express request to begin national examination procedures (35 U S C 371(f)) The submission must include itens (5), (6), (9) and (24) indicated below.
4. ☐ The US has been elected by the expiration of 19 months from the priority date (Article 31)
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   - a. ☐ is attached hereto (required only if not communicated by the International Bureau)
   - b ☐ has been communicated by the International Bureau
   - c ☒ is not required, as the application was filed in the United States Receiving Office (RO/US)
6. ☐ An English language translation of the International Application as filed (35 U S C. 371(c)(2))
   - a. ☐ is attached hereto.
   - b ☐ has been previously submitted under 35 U.S C 154(d)(4)
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   - a. ☐ are attached hereto (required only if not communicated by the International Bureau)
   - b ☐ have been communicated by the International Bureau
   - c ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   - d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S C 371(c)(3))
9. ☒ An oath or declaration of the inventor(s) (35 U S.C. 371 (c)(4))
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409)
12. ☒ A copy of the International Search Report (PCT/ISA/210).

Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3 28 and 3 31 is included
15. ☐ A **FIRST** preliminary amendment
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter
19. ☐ A computer-readable form of the sequence listing in accordance wi
20. ☐ A second copy of the published international application under 35
21. ☐ A second copy of the English language translation of the internatio
22. ☐ Certificate of Mailing by Express Mail
23. ☒ Other items or information:

    1) Courtesy copy of International Application;
    2) Copy of Written Opinion; and
    3) Return post card

"Express Mail" Label No. **EL846059707US**
Date of Deposit **November 5, 2001**

I hereby certify that this paper is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Assistant Commissioner for Patents, Box PCT, Washington, D.C. 20231.

By Deborah Ehret
Typed Name: Deborah Ehret

PCTUS1/REV03

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U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
10/009472	PCT/US00/11893	RU-0170

24. The following fees are submitted .

	CALCULATIONS	PTO USE ONLY
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :		
☐ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO . . . **$1040.00**		
☒ International preliminary examination fee (37 CFR 1 482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$890.00**		
☐ International preliminary examination fee (37 CFR 1 482) not paid to USPTO but international search fee (37 CFR 1 445(a)(2)) paid to USPTO **$740.00**		
☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$710.00**		
☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	**$890.00**	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e))	**$0.00**	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	20 - 20 =	0	x $18.00	**$0.00**	
Independent claims	5 - 3 =	2	x $84 00	**$168.00**	
Multiple Dependent Claims **(check if applicable)**			☐	**$0.00**	
TOTAL OF ABOVE CALCULATIONS =				**$1,058.00**	
☒ Applicant claims small entity status See 37 CFR 1.27). The fees indicated above are reduced by 1/2.				**$529.00**	
SUBTOTAL =				**$529.00**	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). +				**$0.00**	
TOTAL NATIONAL FEE =				**$529.00**	
Fee for recording the enclosed assignment (37 CFR 1 21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **(check if applicable).** ☐				**$0.00**	
TOTAL FEES ENCLOSED =				**$529.00**	
				Amount to be: refunded	$
				charged	$

a. ☐ A check in the amount of ________ to cover the above fees is enclosed.

b. ☒ Please charge my Deposit Account No. **50-1619** in the amount of **$529.00** to cover the above fees A duplicate copy of this sheet is enclosed

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **50-1619** A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

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6/PRTS

COMPOSITIONS AND METHODS FOR DETECTION OF ACTIVE PROTEASES

This application claims priority to U.S. Provisional Application No. 60/132,358, filed May 4, 1999, the entirety of which is incorporated by reference herein.

FIELD OF THE INVENTION

This invention relates to the field of detection and measurement of biological molecules. In particular, the invention provides a novel assay system for detecting the presence or amount of selected active proteases in biological samples.

BACKGROUND OF THE INVENTION

Various scientific articles are referred to in parentheses throughout the specification, and complete citations are listed at the end of the specification. These articles are incorporated by reference herein to describe the state of the art to which this invention pertains.

Proteases are ubiquitous enzymes that play important roles in the control of cellular processes. In eukaryotes, proteases play key roles in orchestrating the progression of the cell cycle as well as in the decision process for activating programmed cell death. For example, it has become clear in the past 10 years that a large number of distinct but related cysteine proteases, called caspases, are involved in cell death activation in animals (Cryns and Yuan, 1998). From gene knock-out studies in mice, it is quite clear that different caspases play distinct roles in the cell death control of various tissues. In addition, although aspartate is the invariant residue at the P1 position of their target sites, animal caspases can be distinguished from each other by their preference of distinct substrate peptide sequences (Talanian et al. 1997). The ability to monitor the presence of different caspases in vivo should greatly facilitate our understanding of how this family of important protease may be controlled at the level of their enzymatic activity.

protein having a detectable biological activity when not fused to the repressor domain; and (c) a protease cleavage domain linking the repressor domain to the reporter domain, the protease cleavage domain comprising a structure that is cleaved by activity of the pre-determined protease. In a preferred embodiment, the repressor domain is a hormone binding domain of a steroid hormone receptor, the reporter domain is β-glucuronidase and the protease cleavage domain is a cleavage site for a caspase.

In another embodiment, the chimeric protease detector protein comprises at least one repressor domain and a plurality of reporter domains, each linked to the repressor domain(s) by a protease cleavage site. Using a multiplicity of reporters and cleavage sites, this protease detector protein can be used to detect more than one selected protease.

According to another aspect of the invention, a method is provided for determining the presence or activity of a pre-determined protease in a biological sample, which utilizes the chimeric protease detector protein described herein. The method comprises adding the protease detector protein to the biological sample suspected of containing the pre-determined protease and measuring the detectable biological activity, if any, of the reporter domain. The occurrence and amount of the detectable biological activity is proportional to the occurrence and amount of the pre-determined protease in the biological sample.

The aforementioned method may be used in a biological sample comprises a biological fluid, tissue or cell extract by providing the protease detector protein as an isolated protein. Alternatively, the method can be used in a biological sample comprising intact cells in which the pre-determined protease, if present, is contained. In this instance, the protease detector protein is provided by introducing into the cells an expressible DNA construct that encodes the protein, under conditions whereby the protein is expressed. The DNA construct may be stably or transiently introduced into the cells.

According to another aspect of the invention, the above-described methods can be adapted for determining the presence or amount of a plurality of pre-determined proteases. This is accomplished by adding a plurality of protease detector

Figure 2. Schematic diagram showing the method for the construction of the fusion proteins described in Example 1. Fig. 2A: shows step1, introduction of the caspase target cleavage site by polymerase chain reaction (PCR). Fig. 2B: shows step 2, creating an intermediate chimeric clone, 3'GUS-YVADG-HBD. Fig. 2C: shows step 3, 5' end GUS gene reconstruction.

Figure 3. Autoradiogram of SDS-PAGE gel demonstrating that the linker site between GUS and GR-HBD can be specifically recognized and cleaved by purified caspase-1. Lefthand 3 lanes show protease detector GUS-YVAD-HBD, in the presence or absence of caspase-1 and/or a peptide inhibitor of caspase-1, AcYVAD-CMK; center 3 lanes show control construct GUS-YVAA-HBD that is not recognized by caspase-1, in the presence or absence of caspase-1 and/or a peptide inhibitor of caspase-1; righthand 3 lanes show GUS alone, in the presence or absence of caspase-1 and/or a peptide inhibitor of caspase-1.

Figure 4. Graphs showing GUS activity from *in vitro* translated samples using various constructs. Fig. 4A shows results of an experiment in which reticulocyte lysate alone, or expressing the caspase-1 detector construct in the presence of caspase, were examined for GUS activity. Fig. 4B shows results of a second experiment that tested GUS activity in *in vitro* translations using a variety of control and test combinations. Legend: Ret = reticulocyte lysate; casp1 = caspase-1; Ac-YVAD-CMK = peptide inhibitor of caspase-1; TGUS-YVAD-HBD = the caspase-1 protease detector; C = conrol (no caspase-1); TGUS-YVAA-HBD = the caspase-1 protease detector control construct.

DETAILED DESCRIPTION OF THE INVENTION

I. Definitions:

Various terms relating to the present invention are used hereinabove and also throughout the specifications and claims.

A "coding sequence" or "coding region" refers to a nucleic acid molecule having sequence information necessary to produce a gene product, when the sequence is expressed.

The term "operably linked" or "operably inserted" means that the

[redacted]

transformed cell.

The term "reporter gene" refers to a gene that encodes a product which is easily detectable by standard methods, either directly or indirectly.

A "heterologous" region of a nucleic acid construct is an identifiable segment (or segments) of the nucleic acid molecule within a larger molecule that is not found in association with the larger molecule in nature. Thus, when the heterologous region encodes a mammalian gene, the gene will usually be flanked by DNA that does not flank the mammalian genomic DNA in the genome of the source organism. In another example, coding sequence is a construct where the coding sequence itself is not found in nature (e.g., a cDNA where the genomic coding sequence contains introns, or synthetic sequences having codons different than the native gene). Allelic variations or naturally-occurring mutational events do not give rise to a heterologous region of DNA as defined herein.

A cell has been "transformed" or "transfected" by exogenous or heterologous DNA when such DNA has been introduced inside the cell. The transforming DNA (transgene) may or may not be integrated (covalently linked) into the genome of the cell. In prokaryotes, yeast, and mammalian cells for example, the transforming DNA may be maintained on an episomal element such as a plasmid. With respect to eukaryotic cells, a stably transformed cell is one in which the transforming DNA has become integrated into a chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing the transforming DNA. A "clone" is a population of cells derived from a single cell or common ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth *in vitro* for many generations. If germline cells are stably transformed, the transformation may be passed from one generation of animals arising from the germline cells, to the next generation. In this instance, the transgene is referred to as being inheritable.

Other definitions are found in the description set forth below.

Sambrook et al., Molecular Cloning, Cold Spring Harbor Laboratory (1989) (hereinafter "Sambrook et al.") or Ausubel et al. (eds) Current Protocols in Molecular Biology, John Wiley & Sons (2000) (hereinafter "Ausubel et al.") are used.

The detection system of the invention utilizes a three-part chimeric protein, referred to herein as a "protease detector". The protease detector is composed of (1) a "repressor domain"; (2) a "reporter domain" comprising an enzyme that, when liberated from the repressor domain, has activity which is easily detectable; and (3) a protease cleavage domain that joins the repressor domain to the reporter domain. The protease detector is introduced into a test sample containing, or suspected of containing, a protease that specifically recognizes the protease cleavage domain. If present, the protease cleaves the chimeric detector at the cleavage domain, thereby liberating the reporter domain and allowing it to become active. Enzymatic activity of the reporter domain is detected, and the presence or amount of that activity is correlated to the presence or amount of the protease in the test sample.

The repressor domain can be any protein domain that represses the activity of a reporter enzyme to which it is linked via the protease cleavage domain. In preferred embodiments, repressor domains are taken from cellular receptors whose activity in cells is repressed until activated by binding of its cognate ligand. In accordance with the present invention, however, it has been found that, these repressor domains can act as ligand-independent repressors of activity of enzymes linked to them via a protease cleavage domain. Thus, the present invention differs in its fundamental nature from assays using ligand-activated receptors such as HBD, in that this system does not need to be activated by ligand binding to the receptor. Activation takes place only after protease cleavage.

The steroid hormone receptors are examples of cellular receptors whose binding domains provide particularly suitable repressor domains for use in the protease detector proteins of the invention. The steroid hormone receptors are members of a large family of important transcriptional regulators in animal systems. These proteins functions to transduce signals from steroid hormones to control cellular processes via the control of gene expression in the nucleus. The hormone binding domain (HBD) of the steroid receptor acts as a regulatory domain to control

detectable. Detectability can be by any means, but preferably relates to biological activity that is regained upon liberation of the reporter domain from the repressor domain. Examples of reporter domains suitable for use in the present invention include, but are not limited to, β-glucuronidase (GUS), β-galactosidase, chloramphenicol acetyl transferase (CAT), various transcription factors, alcohol dehydrogenase and luciferase.

The protease cleavage domain links the repressor domain to the reporter domain. This domain comprises a peptide sequence specifically recognized and cleaved by the protease whose activity is being assayed. A variety of proteases recognize specific cleavage sites in polypeptide sequences. Examples are set forth in the table below, which contains a list of characterized proteases and their specific substrates. Cleavage takes place between amino acid residue X and the P1 position for each of the target sites (X represents any amino acid) (Source: http//delphi. phys.univ-tours.fr/ Prolysis/sublist.html and the catalog from Calbiochem Co.).

Protease	Target site		
	3-letter code	1-letter code	SEQ ID NO:
Calpain	Val-Leu-Lys-X	VLKX	10
Cathepsin G	Ala-Val-Pro-Phe-X	AVPFX	11
Collagenase	Pro-Gln-Gly-Ile-Ala-Gly-Gln-X	PQGIAGQX	12
Elastase I	Ala-Ala-Pro-Val-X	AAPVX	13
Elastase II	Ala-Ala-Pro-Ala-X	AAPAX	14
Granzyme B	Ala-Ala-Asp-X	AADX	15
MMP-1	Pro-Gln-Gly-Ile-Ala-Gly-Gln-DArg-X	PGGIAGQrX	16
Kallikrein	Pro-Phe-Arg-X	PFRX	17
Papain	Gln-Val-Val-Ala-		

City, CA), according to manufacturer's instructions. Other methods of synthesizing peptides or peptidomimetics, either by solid phase methodologies or in liquid phase, are well known to those skilled in the art. When solid-phase synthesis is utilized, the C-terminal amino acid is linked to an insoluble resin support that can produce a detachable bond by reacting with a carboxyl group in a C-terminal amino acid. One preferred insoluble resin support is *p*-hydroxymethylphenoxymethyl polystyrene (HMP) resin. Other useful resins include, but are not limited to: phenylacetamidomethyl (PAM) resins for synthesis of some N-methyl-containing peptides (this resin is used with the Boc method of solid phase synthesis; and MBHA (p-methylbenzhydrylamine) resins for producing peptides having C-terminal amide groups.

During the course of peptide synthesis, branched chain amino and carboxyl groups may be protected/deprotected as needed, using commonly-known protecting groups. In a preferred embodiment, N^{α}-amino groups are protected with the base-labile 9-fluorenylmethyloxycarbonyl (Fmoc) group or *t*-butyloxycarbonyl (Boc groups). Side-chain functional groups consistent with Fmoc synthesis may be protected with the indicated protecting groups as follows: arginine (2,2,5,7,8-pentamethylchroman-6-sulfonyl); asparagine (O-t-butyl ester); cysteine glutamine and histidine (trityl); lysine (*t*-butyloxycarbonyl); serine and tyrosine (*t*-butyl). Modification utilizing alternative protecting groups for peptides and peptide derivatives will be apparent to those of skill in the art.

Full-length proteins or protein domains for use in the present invention may be prepared in a variety of ways, according to known methods. Proteins may be purified from appropriate sources, e.g., human or animal cultured cells or tissues, by various methods such as gel filtration, ion exchange chromatography, reverse-phase HPLC and immunoaffinity purification, among others. However, due to the often limited amount of a protein present in a sample at any given time, conventional purification techniques are not preferred in the present invention.

The availability of nucleic acids molecules encoding a protein enables production of the protein using *in vitro* expression methods known in the art. For example, a cDNA or gene may be cloned into an appropriate *in vitro* transcription

coupled to a protein using glutaraldehyde, a common cross-linking agent. Another method for chemically coupling a peptide to a protein is through the use of carbodiimide and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide methiodide (EDC). Methods for joining two proteins together are also available.

The peptides or proteins of the invention, prepared by the aforementioned methods, may be analyzed according to standard procedures. For example, they may be subjected to amino acid sequence analysis, mass spectra analysis or amino acid compositional analysis according to known methods.

The chimeric protease detector proteins of the present invention comprise two domains linked together by a protease cleavage site with or without linkers. The organization of the respective domains can differ. For instance, if "R" represents the repressor domain, "D" represent the detectable reporter domain, and "C" represents the protease cleavage domain (with or without linkers), the protease detector proteins of the present invention may be organized as follows:

D - C - R;

R - C - D;

D_1 - C_1 - R - C_2 - D_2; wherein subscripted numbers indicated different cleavage sites or reporter domains. It should also be apparent that multiple repressor domains can be used in designing a complex protease detector protein.

The protease detector proteins of the present invention can be used singly or in combination to detect and quantitate activity of selected proteases *in vitro* and *in vivo*. The proteins can be used to assay various biological fluids, including tissue or cell extracts or environmental samples for activity of pre-determined proteases. Furthermore, *in vitro* detection of protease activation will enable the screening for drugs that affect cellular processes where proteases are activated either directly or upstream in a signal transduction pathway. The addition of appropriate substrates for the particular reporter enzyme in a microtiter plate will enable one to correlate protease activation with reporter enzyme activity. In the reverse approach, it can allow one to detect the presence of protease inhibitors or inhibitors of upstream components of the protease signaling pathway which normally leads to protease

comprises a chimeric protease detector protein as describe above, along with instructions on how to use the protein to detect the presence or activity of a pre-determined protease and, optionally, further comprises at least one other reagent useful for conducting assays to detect the presence or activity of a protease. In a particularly preferred embodiment, the test kit is adapted for detection of a plurality of pre-determined proteases, and comprises two or more different protease detector proteins.

The invention provides another test kit useful for continuous monitoring of protease activity in a selected cell type. This kit provides a protease detector system having two constructs; one in which a transcription factor is linked to a repressor moiety via the protease cleavage site, and the other comprising a reporter gene under the control of a promoter and the DNA responsive element activated by binding of the transcription factor. This kit also may provide selected cell types for practicing the assay, along with various reagents for culturing the cells, introducing the constructs into the cells, and detecting expression of the reporter gene.

The following example is provided to describe the invention in greater detail. It is intended to illustrate, not to limit, the invention.

EXAMPLE 1

HBD/GUS Construct for Detecting Active Caspase

In this example, it is experimentally determined whether the HBD of GR and other steroid receptors could possibly act as general repressor domains that could be used to mask the enzyme activities of a protein fusion partner. The placement of a defined protease target site sequence between the two partners would then release the latent enzymatic activity from repression by the HBD and the associated HSP90.

A prerequisite for this strategy is the accessibility of the protease target site in the fusion protein and the ability of the released enzyme to regain its active state. Prior to the experimental results reported herein, it was entirely unclear whether the cleavage site would be available or, if available, if the released reporter enzyme

constructs while the GUS alone construct produced a protein with an apparent mass of about 70 kDa. Addition of purified caspase-1 generated cleavage products of 70 kDa and about 30 kDa from the YVADG containing fusion but not the P1 linker variant. In fact, no obvious proteolysis was detected by caspase-1 was observed with either the P1 linker mutant fusion or the GUS alone control. As expected, the cleavage of the YVADG containing fusion protein by caspase-1 can be inhibited by addition of the caspase specific peptide inhibitor YVAD-cmk.

To assay for the activity of the fusion proteins, we carried out *in vitro* transcription/translation of the different constructs without radiolabelled methionine. The results are presented in Figure 4. In the absence of caspase-1, either fusion protein show essential no significant GUS activity above the low background present in reticulocyte lysates alone. In a separate experiment, we found that transcription/translation with the GUS alone control can produce high GUS activity with this assay system (data not shown). Thus, the absence of GUS activity with the fusion proteins is likely due to the inactivation of the GUS partner by its fusion to the GR-HBD domain and not due to the presence of inhibitory compounds in the lysate. Upon addition of caspase-1 to the YVADG containing fusion protein, a dramatic appearance of GUS is observed. This is in contrast to the YVAAG containing fusion protein, in which case no detectable increase in GUS activity is observed. The unmasking of the GUS activity in the fusion is due to the proteolytic cleavage by the added caspase-1 since inclusion of the caspase specific inhibitor YVAD-cmk abolished this process. These results demonstrate that the released GUS enzyme partner can function properly as a reporter of active caspase cleavage.

In this example, we documented the successful application of the steroid hormone receptor as a sensitive reporter system to detect active protease *in vitro*. Using GUS as a model reporter, we found that fusion of the GR-HBD can effectively mask the intrinsic enzyme activity of the GUS partner in the chimeric protein produced in the rabbit reticulocyte lysate system. Quantitative kinetic analysis of GUS enzyme activity demonstrated no detectable cleavage of the fluorogenic substrate 4-MUG by the fusions. The tightness of this repression by the HBD domain is likely linked to the fact that the GUS protein needs to dimerize in order to form the

Heim R and Tsien RY (1996) Engineering green fluorescent protein for improved brightness, longer wavelengths and fluorescence resonance energy transfer. Curr. Biol 6: 178-182.

Mattioni T, Louvion J-F and Picard D (1994) Regulation of protein activities by fusion to steroid binding domains. in Methods in Cell Biology 43: 335-352.

Simon R., Igeno MI and Coupland G (1996) Activation of floral meristem identity gene in Arabidopsis. Nature 384: 59-62.

Talanian RV, Quinlan C, Trautz S, Hackett MC, Mankovich JA, Banach D, Ghayur T, Brady KD and Wong WW (1997) Substrate specificities of caspase (interleukin-1b converting enzyme) family proteases. J. of Biol. Chem. 272: 9677-9682.

The present invention is not limited to the embodiments described and exemplified above, but is capable of variation and modification without departure from the scope of the appended claims.

8. The chimeric protein of claim 7, wherein the protease cleavage sites are different from one another.

9. A chimeric protein for measuring caspase activity, comprising a hormone binding domain linked to a β-glucuronidase enzyme by a peptide comprising a caspase cleavage site, wherein the β-glucuronidase is inactive due to linkage to the hormone binding domain and release of the β-glucuronidase through caspase cleavage of the cleavage site restores activity of the β-glucuronidase.

10. A method for determining the presence or activity of a pre-determined protease in a biological sample, which comprises:

a) providing a chimeric protease detector protein comprising:

i) a repressor domain which represses activity of a normally biologically active protein fused thereto;

ii) a reporter domain comprising a protein having a detectable biological activity when not fused to the repressor domain; and

iii) a protease cleavage domain linking the repressor domain to the reporter domain, the protease cleavage domain comprising a structure that is cleaved by activity of the pre-determined protease;

b) adding the protease detector protein to the biological sample suspected of containing the pre-determined protease; and

c) measuring the detectable biological activity, if any, of the reporter domain, the occurrence and amount of the detectable biological activity being proportional to the occurrence and amount of the pre-determined protease in the biological sample.

11. The method of claim 10, wherein the biological sample comprises a biological fluid, tissue or cell extract and the protease detector protein is provided as an isolated protein.

12. The method of claim 10, wherein the biological sample comprises

domain to the reporter domain, the protease cleavage domain comprising a structure that is cleaved by activity of the pre-determined protease;

b) preparing a test sample and a control sample, the test sample containing the pre-determined protease, the protease detector protein and the test compound, the control sample containing the pre-determined protease and the protease detector protein;

c) measuring the detectable biological activity, if any, of the reporter domain, in the test sample and the control sample; and

d) comparing the amount of the detectable biological activity in the test sample with that in the control sample, an increase or decrease of the activity in the test sample being indicative of the ability of the test compound to affect the amount or activity of the protease.

19. A test kit for detecting the presence or activity of a pre-determined protease, which comprises a container containing:

a) a chimeric protease detector protein comprising:

i) a repressor domain which represses activity of a normally biologically active protein fused thereto;

ii) a reporter domain comprising a protein having a detectable biological activity when not fused to the repressor domain; and

iii) a protease cleavage domain linking the repressor domain to the reporter domain, the protease cleavage domain comprising a structure that is cleaved by activity of the pre-determined protease;

b) optionally, at least one other reagent for using the protease detector protein; and

c) optionally, instructions for using the protease detector protein.

20. The test kit of claim 19, adapted for detection of a plurality of pre-determined proteases, which comprises a plurality of chimeric protease detector proteins.

N/e

PCT

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International Bureau

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(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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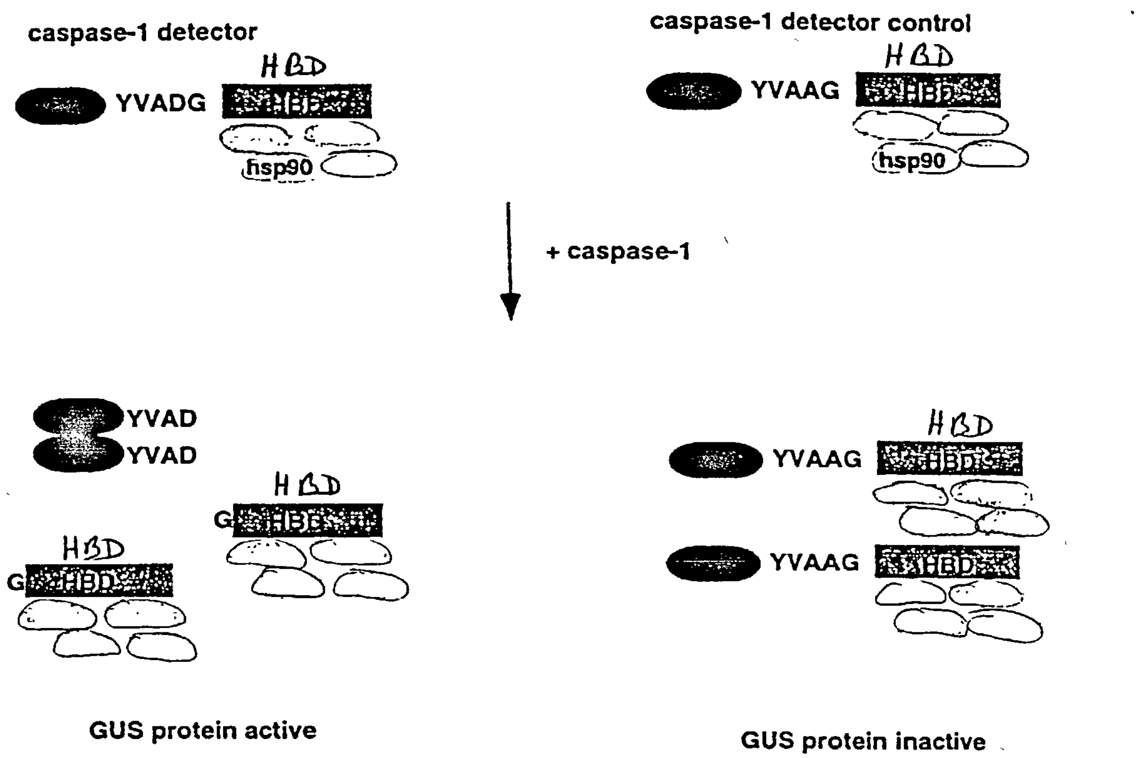
(54) Title: COMPOSITIONS AND METHODS FOR DETECTION OF ACTIVE PROTEASES

(57) Abstract

A novel assay system is disclosed for detecting the presence or amount of selected active proteases in biological samples. The assay system utilizes a chimeric protease detector protein composed of three domains: (1) a repressor domain, (2) a protease cleavage domain specific for the protease to be assayed, and a reporter domain. The reporter domain is not detectable when linked to the repressor domain, but becomes detectable upon release from the repressor domain by protease-mediated cleavage. Thus, the activity of the selected protein can be determined by measuring the amount of detectable reporter in the sample. Methods and test kits for using the novel assay system in a variety of *in vitro* and *in vivo* applications are also disclosed.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HBD
YVADG
HBD
hsp90
caspase-1 detector control
HBD
YVAAG
HBD
hsp90
+ caspase-1
YVAD
YVAD
HBD
G
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HBD
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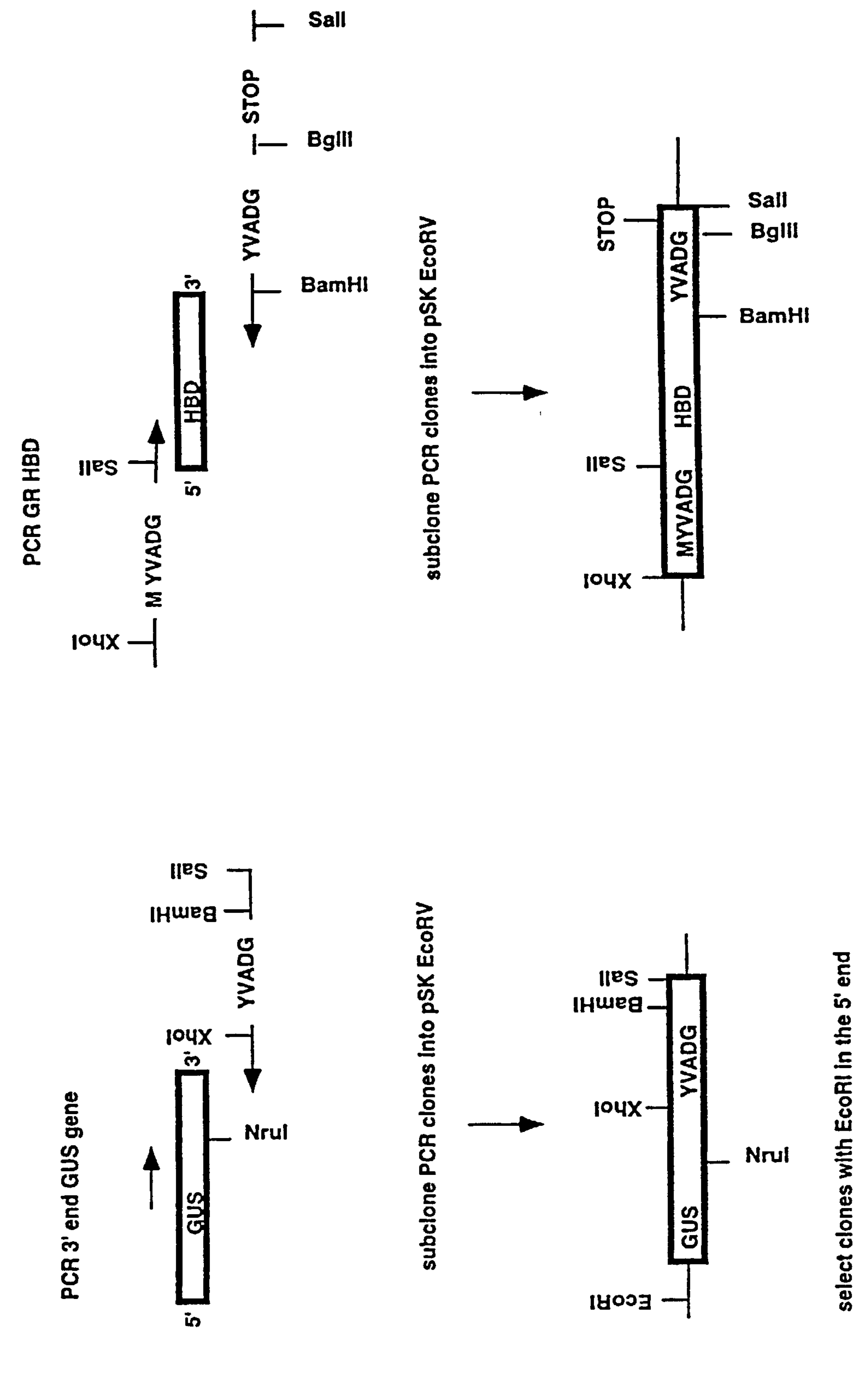
HBD
GUS protein active
GUS protein inactive

Fig.1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M YVADG
SalI
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HBD
3'
BamHI
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STOP
SalI
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XhoI
MYVADG
SalI
HBD
YVADG
BamHI
BglII
STOP
SalI
PCR 3' end GUS gene
5'
GUS
3'
NruI
XhoI
YVADG
BamHI
SalI
subclone PCR clones into pSK EcoRV
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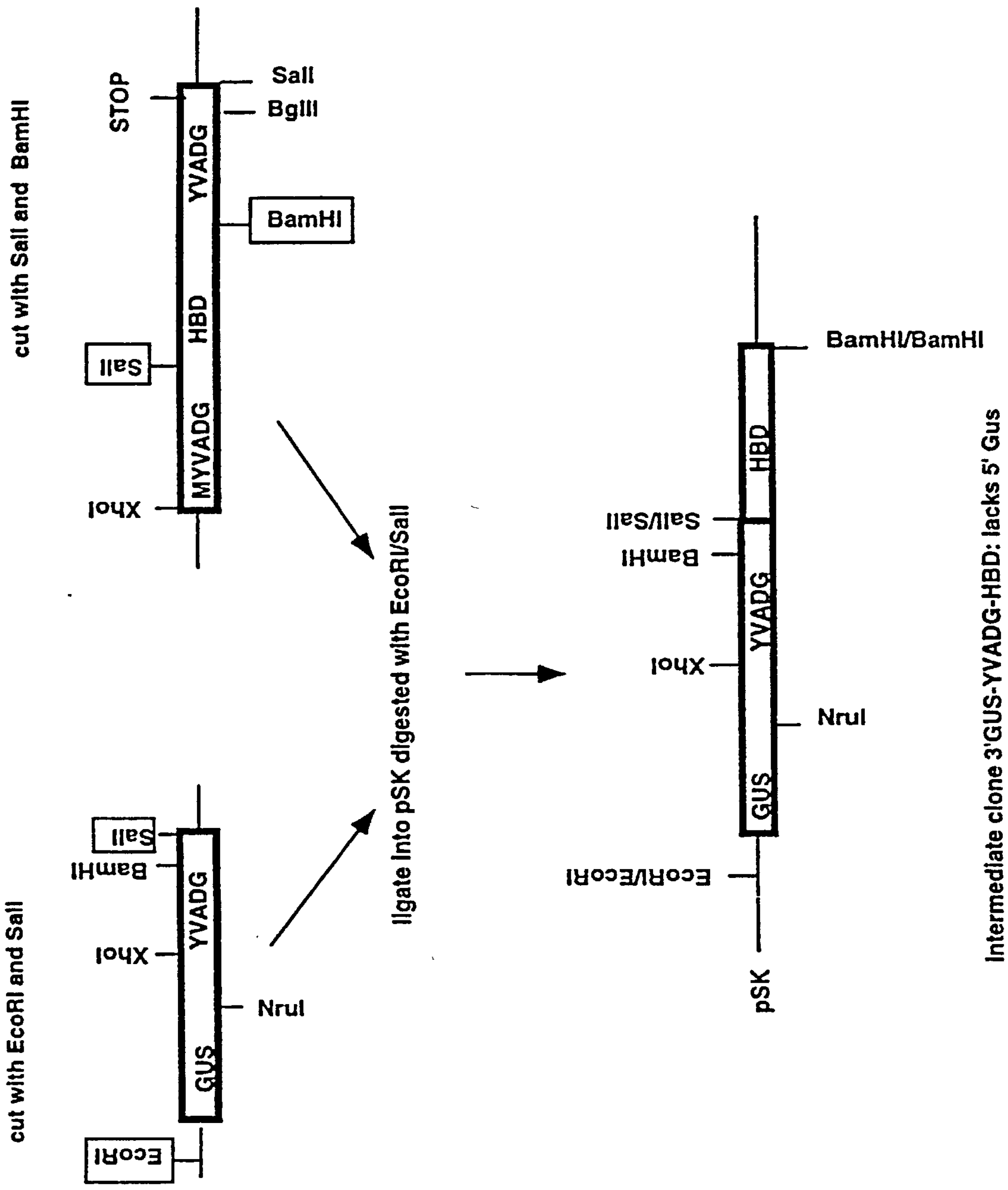
SalI
select clones with EcoRI in the 5' end

Fig.2a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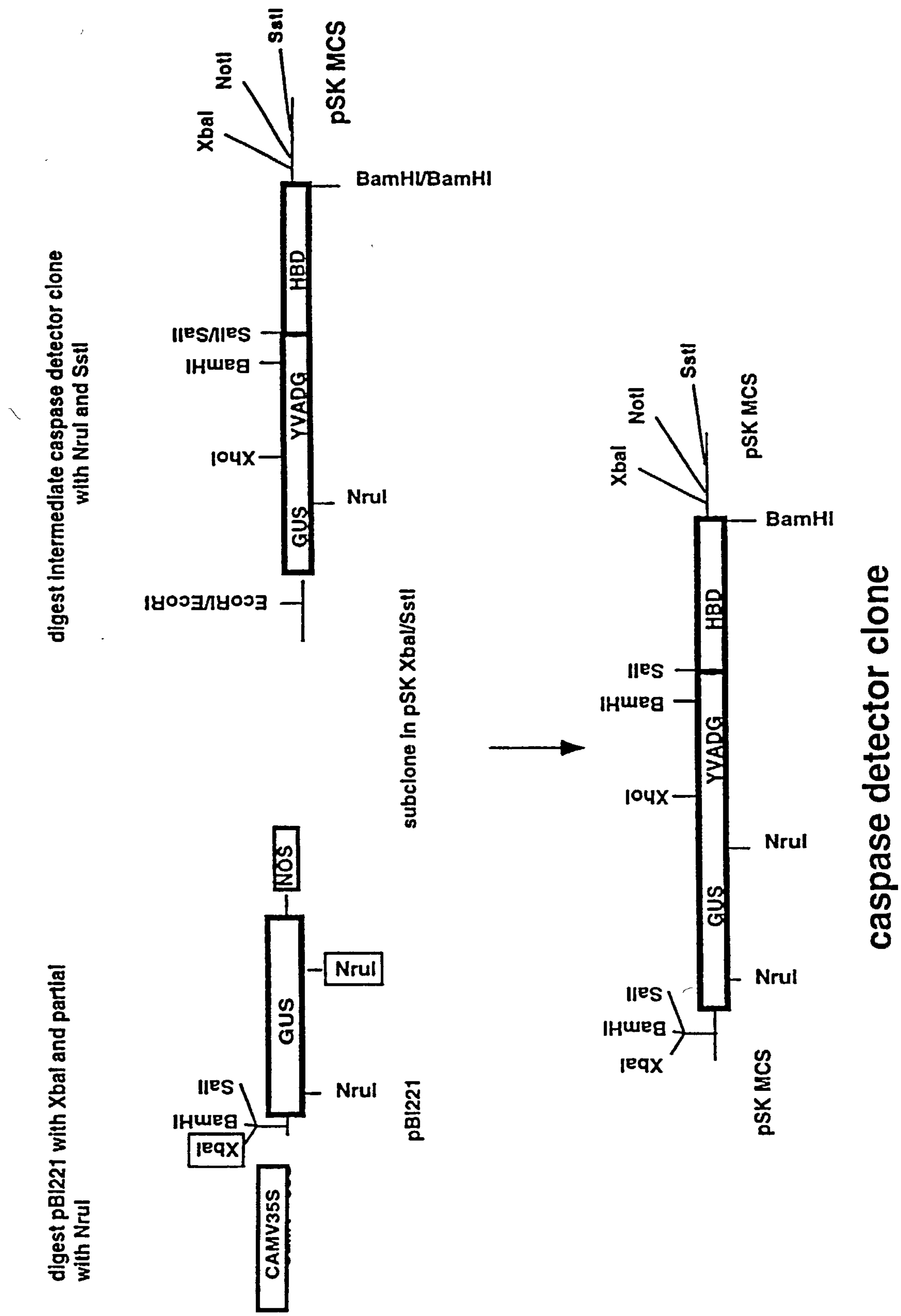
HBD
SalI
MYVADG
XhoI
cut with EcoRI and SalI
SalI
BamHI
YVADG
XhoI
NruI
GUS
EcoRI
ligate into pSK digested with EcoRI/SalI
BamHI/BamHI
HBD
SalI/SalI
BamHI
YVADG
XhoI
NruI
GUS
EcoRI/EcoRI
pSK
Intermediate clone 3'GUS-YVADG-HBD: lacks 5' Gus

Fig.2b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NotI
SstI
pSK MCS
BamHI/BamHI
HBD
SalI/SalI
BamHI
YVADG
XhoI
GUS
NruI
EcoRI/EcoRI
subclone in pSK XbaI/SstI
digest pBI221 with XbaI and partial with NruI
NOS
GUS
NruI
SalI
BamHI
XbaI
NruI
CAMV35S
pBI221
XbaI
NotI
SstI
pSK MCS
BamHI
HBD
SalI
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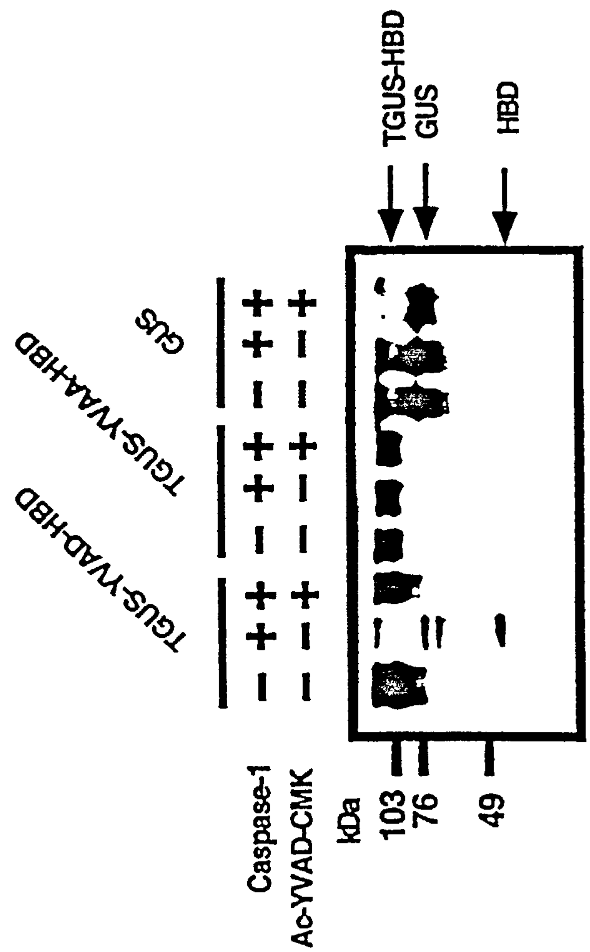
pSK MCS
caspase detector clone

Fig.2c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Caspase-1
Ac-YVAD-CMK
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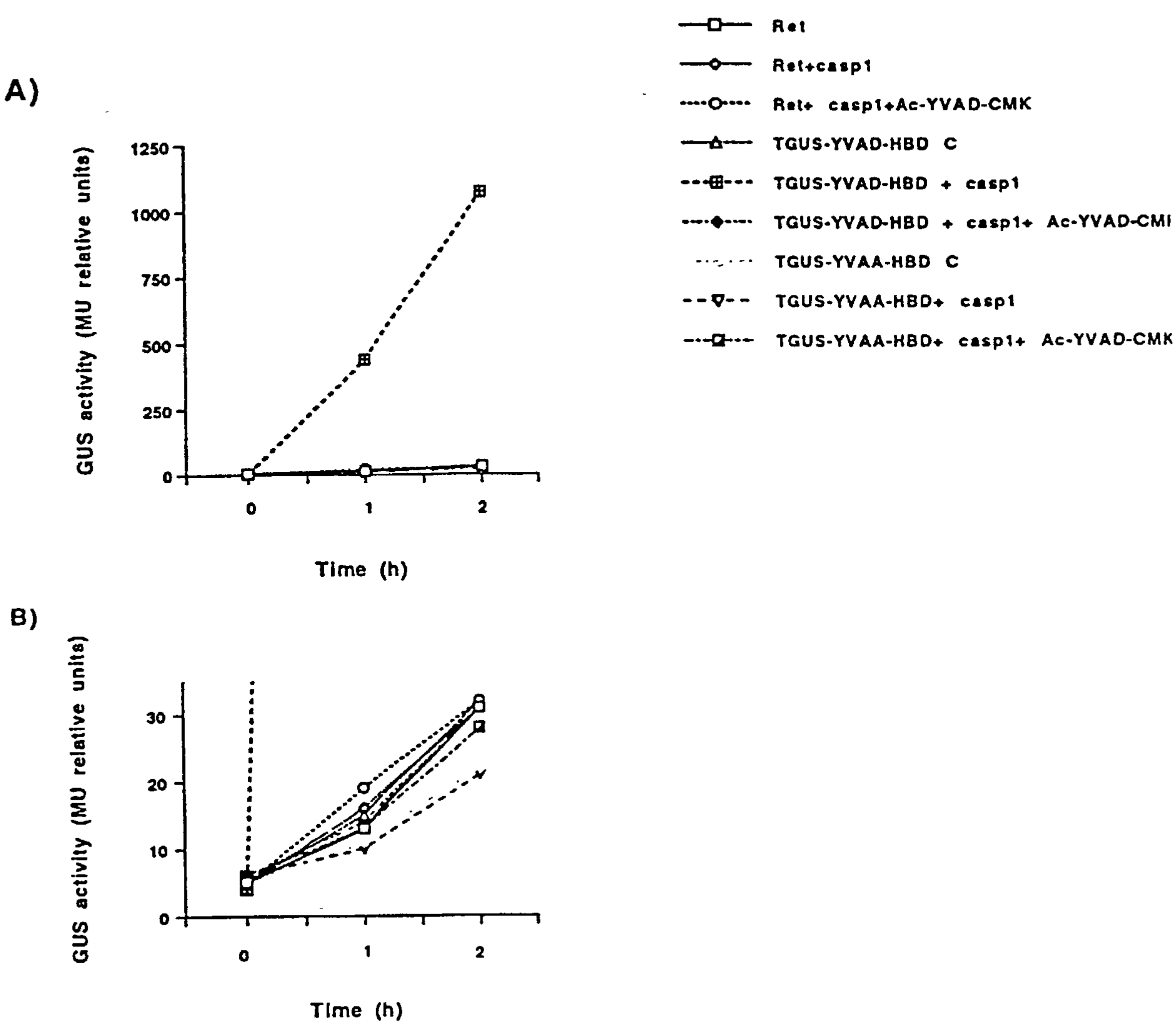
76
49
TGUS-HBD
GUS
HBD

Fig.3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2
Time (h)
B)
GUS activity (MU relative units)
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20
10
0
0
1
2
Time (h)
Ret
Ret+casp1
Ret+ casp1+Ac-YVAD-CMK
TGUS-YVAD-HBD C
TGUS-YVAD-HBD + casp1
TGUS-YVAD-HBD + casp1+ Ac-YVAD-CMI
TGUS-YVAA-HBD C
TGUS-YVAA-HBD+ casp1
TGUS-YVAA-HBD+ casp1+ Ac-YVAD-CMK

Fig.4

Docket No.
RU-0170

10/009412

Declaration and Power of Attorney For Patent Application

English Language Declaration

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

Compositions and Methods for Detection of Active Proteases

the specification of which

(check one)

☐ is attached hereto.

☒ was filed on 2 May 2000 as United States Application No. or PCT International Application Number PCT/US00/11893 and was amended on ______________________

(if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)			Priority Not Claimed
(Number)	(Country)	(Day/Month/Year Filed)	☐
(Number)	(Country)	(Day/Month/Year Filed)	☐
(Number)	(Country)	(Day/Month/Year Filed)	☐

I hereby claim the benefit under 35 U.S.C. Section 119(e) of any United States provisional application(s) listed below:

60/132,358	**May 4, 1999**
(Application Serial No.)	(Filing Date)
(Application Serial No.)	(Filing Date)
(Application Serial No.)	(Filing Date)

I hereby claim the benefit under 35 U. S. C. Section 120 of any United States application(s), or Section 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. Section 112, I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, C. F. R., Section 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. *(list name and registration number)*

26259
PATENT TRADEMARK OFFICE

Send Correspondence to:

Direct Telephone Calls to: *(name and telephone number)*
Jane Massey Licata or Kathleen A. Tyrrell - (856) 810-1515

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Sole or first inventor's signature	Date Nov. 24, 2001
Residence **East Brunswick, New Jersey** NJ	
Citizenship **US**	
Post Office Address **19 Corona Road**	
East Brunswick, New Jersey 08816	

Full name of second inventor, if any **Olga del Pozo**	Nov. 28 2001
Second inventor's signature	Date
Residence **Sevilla, Spain** ESX	
Citizenship **Spain**	
Post Office Address **Felipe II , no. 21**	
Sevilla 41013 Spain	

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